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Abstract: RAGE is a central driver of tumorigenesis by sustaining an inflammatory tumor microenvironment. This study links the soluble forms of RAGE (sRAGE and esRAGE) with clinical outcome of melanoma patients. Moreover, tissue expression of RAGE was analyzed using immunohistochemistry on two independent tissue microarrays (TMA) containing 35 or 257 primary melanomas, and 41 or 22 benign nevi, respectively. Serum concentrations of sRAGE and esRAGE were measured in 229 stage III-IV patients using ELISA and plasma concentrations of sRAGE were analyzed in an independent second cohort with 173 samples of stage I-IV patients. In this cohort, three well-described SNPs in the RAGE gene were analyzed. RAGE protein expression was highly upregulated in primary melanomas compared to benign nevi in the two TMA ($p < 0.001$ and $p = 0.005$) as well as in sun-exposed melanomas ($p = 0.046$). sRAGE and esRAGE were identified as prognostic markers for survival as diminished sRAGE ($p = 0.034$) and esRAGE ($p = 0.012$) serum levels correlated with poor overall survival (OS). Multivariate Cox regression analysis showed that diminished serum sRAGE was independently associated with poor survival ($p = 0.009$). Moreover, diminished sRAGE was strongly associated with impaired OS in the second cohort ($p < 0.001$). Multivariate Cox regression analysis including the investigated SNPs revealed an independent correlation of the two interacting promoter SNPs with impaired OS. In conclusion, the soluble forms of RAGE and variants in its genetic locus are prognostic markers for survival in melanoma patients with high risk for progression.

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Soluble RAGE and RAGE polymorphisms in melanoma

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ABBREVIATIONS

ADAM10: a disintegrin and metalloproteinase domain-containing protein 10; AJCC: American joint committee on cancer; DAMP: damage-associated molecular pattern; ELISA: enzyme-linked immunosorbent assay; LDH: lactate dehydrogenase; MMP-9: matrix metallopeptidase 9; OS: overall survival; RAGE: receptor for advanced glycation end-products; esRAGE: endogenous secretory receptor for advanced glycation end-products; sRAGE: soluble receptor for advanced glycation end-products; RFLP: restriction fragment length polymorphism; ROC: receiver operating characteristic; SNP: single nucleotide polymorphism; TMA: tissue microarray; ULN: upper limit of normal

NOVELTY AND IMPACT

The aptly named RAGE protein fuels chronic inflammation. In healthy persons, however, soluble forms of RAGE (sRAGE) are abundant in the serum, where they possibly serve as decoy receptors to prevent proinflammatory signaling. Here, among stage III-IV melanoma patients, decreased serum levels of both sRAGE and the splice variant esRAGE were correlated with poor overall survival. By comparison, RAGE protein expression was significantly elevated within primary melanoma tissue. The results suggest that RAGE signaling influences the advance of melanoma and that sRAGE and esRAGE are independent survival indicators in patients at high risk of progression.

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FOOTNOTE

The authors indicated no potential conflicts of interest.

Soluble RAGE and RAGE polymorphisms in melanoma

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Soluble RAGE and RAGE polymorphisms in melanoma

ABSTRACT

RAGE is a central driver of tumorigenesis by sustaining an inflammatory tumor microenvironment. This study links the soluble forms of RAGE (sRAGE and esRAGE) with clinical outcome of melanoma patients. Moreover, tissue expression of RAGE was analyzed using immunohistochemistry on two independent tissue microarrays (TMA) containing 35 or 257 primary melanomas, and 41 or 22 benign nevi, respectively. Serum concentrations of sRAGE and esRAGE were measured in 229 stage III-IV patients using ELISA and plasma concentrations of sRAGE were analyzed in an independent second cohort with 173 samples of stage I-IV patients. In this cohort, three well-described SNPs in the RAGE gene were analyzed. RAGE protein expression was highly upregulated in primary melanomas compared to benign nevi in the two TMA ($p < 0.001$ and $p = 0.005$) as well as in sun-exposed melanomas ($p = 0.046$). sRAGE and esRAGE were identified as prognostic markers for survival as diminished sRAGE ($p = 0.034$) and esRAGE ($p = 0.012$) serum levels correlated with poor overall survival (OS). Multivariate Cox regression analysis showed that diminished serum sRAGE was independently associated with poor survival ($p = 0.009$). Moreover, diminished sRAGE was strongly associated with impaired OS in the second cohort ($p < 0.001$). Multivariate Cox regression analysis including the investigated SNPs revealed an independent correlation of the two interacting promoter SNPs with impaired OS. In conclusion, the soluble forms of RAGE and variants in its genetic locus are prognostic markers for survival in melanoma patients with high risk for progression.

INTRODUCTION

Melanoma initiation, growth and progression have been associated with micro-environmental factors which orchestrate tumor-stroma interactions.[1,2] However, the importance of these factors for predicting disease progression has not yet been established in a clinical setting. In the clinical care of melanoma patients, early detection of disease progression by biomarkers is crucial to guide treatment decisions and to improve outcome, especially in asymptomatic patients with advanced melanoma (stage III or IV following the criteria of the American Joint Committee on Cancer [AJCC]). Several serological biomarkers have been described to determine the prognosis of patients with distant melanoma metastases.[3,4] Among them, S100B and lactate dehydrogenase (LDH) are most widely used in clinical practice. However, only LDH has been included into the AJCC staging system for patients with stage IV melanoma. Moreover, the standard melanoma biomarker S100B lacks sensitivity to detect melanoma progression in up to 20% of patients with asymptomatic but image-detected metastases.[5] Consequently, there remains a high demand for new, valid markers that predict the risk of disease progression, particularly in the case of loco-regional and distant metastasis with low or even no tumor burden.

Previously, we have demonstrated that the receptor for advanced glycation end-products (RAGE) is a driver of inflammation-associated tumorigenesis by sustaining a chronic inflammatory tumor microenvironment.[6] The RAGE molecule belongs to the immunoglobulin superfamily and represents a membrane-bound pattern-recognition receptor for alarmins.[7,8] Interaction between RAGE and its ligands amplifies the pro-inflammatory response.[7] A soluble form of RAGE (sRAGE) is known to be generated by shedding *via* ADAM10 or by alternative splicing[9] and is abundant in plasma/serum of healthy persons, where it is thought to act as decoy for its ligands.[7] Total sRAGE mainly consists of both sRAGE cleaved from full-length RAGE and esRAGE, a splice variant representing only 20-25 % of total sRAGE levels.[9-11] RAGE-binding alarmins, also known as damage-associated molecular pattern molecules (DAMPs), are released upon damage or cellular stress and are able to activate

immune responses similar to cytokines.[12-14] The melanoma biomarker S100B belongs to this group of danger signaling molecules and is known to interact with RAGE.[12,15]

Recently, three naturally occurring, functional genetic variants within the AGER locus encoding for RAGE have been studied. The single nucleotide polymorphisms (SNPs) -429T/C (rs1800625) and -374T/A (rs1800624) are located in the promoter region and have been shown to increase transcriptional activity of RAGE.[16-18] Presence of these promoter SNPs correlates with higher disease activity of various inflammatory diseases such as rheumatoid arthritis or Crohn's disease.[19] Furthermore, the SNP G82S in exon 3 affects glycosylation of the receptor molecule and thereby enhances ligand binding.[16,20] The G82S polymorphism is associated with higher activity of various diseases such as Alzheimer's disease[21] and correlates with higher risk for diabetic complications.[22] To date, the functional role of these well-described SNPs within the genetic locus of RAGE have not been studied in melanoma patients.

The aim of this study was to investigate soluble forms of RAGE as putative biomarkers for human malignant melanoma. Therefore, measurements of sRAGE in two independent cohorts of patients were conducted and the impact of sRAGE on survival was assessed. Moreover, the genotypes of the three functional RAGE polymorphisms -429T/C, -374T/A, and G82S were determined and their prognostic impact for high-risk melanoma patients was evaluated.

MATERIALS AND METHODS

Patients

For this study, two independent sets of plasma and serum samples from 402 melanoma patients (stages I-IV) were analyzed (Table 2). Cohort 1 (marker identification set) consisted of 229 stage III/IV patients who had been seen between 2008 and 2013 at the Department of Dermatology of the University Hospital Tübingen, Germany. Cohort 2 (marker evaluation set) consisted of 173 stage I-IV patients who had been seen between 2010 and 2012 at the Department of Dermatology of the University Hospital Heidelberg, Germany. Median follow-up was 35.5 months for cohort 1 and 13.8 months for cohort 2. Follow-up time was defined from the date of blood withdrawal to the date of last follow-up or death. Primary endpoint was overall survival (OS). Patients who were alive at the date of last follow-up were censored. Samples were selected according to the following criteria: histologically confirmed melanoma, complete documentation of medical history, primary tumor characteristics, course of the disease, and follow-up. The collection of plasma or serum and documentation of clinical data were performed after patients' informed consent with institutional review board approval. Disease staging was done according to the criteria of the AJCC.[23] Detailed patient characteristics are presented in Table 2. Blood draw was done using gel-coated serum tubes or EDTA-coated plasma tubes (Sarstedt). After centrifugation, serum and plasma was stored at -20 or -80 °C, respectively.

Determination of circulating levels of sRAGE and esRAGE

Concentration of circulating levels of RAGE (sRAGE) and of the endogenous secretory splice variant esRAGE were measured in duplicate using sandwich ELISA kits (R&D Systems, Minneapolis, MN and B-Bridge International, Tokyo, Japan). S100B serum levels had been determined routinely during regular follow-up with Elecsys S100 immunoassay (Roche Diagnostics AG, Rotkreuz, Switzerland, ULN = 0.1 ng/mL) according to the instructions of the manufacturer, and LDH was determined by conventional central laboratory analysis with an ULN = 250 U/L.

Receptor-Ligand interaction *in vitro*

Freshly collected blood from healthy donors was centrifuged at 3,000 U/min for 15 min. Serum was harvested and incubated with bovine S100B (Merck Chemicals Ltd., Nottingham, UK) at different final concentrations ranging from 0 mg/L to 100 mg/L at 37°C for 24h. After incubation, sRAGE levels were determined *via* ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Tissue Microarray (TMA)

Two independent TMAs were conducted using formalin fixed and paraffin embedded tissue according to a previous report (Table 1).[24] Briefly, tissue punch samples (0.6 mm diameter) were taken from tumor or nevus tissue, respectively, and collected in a single TMA block each. TMA 1 contained 152 valid tissue punches from 36 primary melanomas (35 were evaluable) and 41 associated benign nevi in duplicate punches from 26 patients. TMA 2 contained 279 valid tissue punches from 257 primary melanomas and 22 benign nevi in single punches from 279 patients.

Immunohistochemical staining was performed as described previously.[6] Briefly, after deparaffinization and antigen retrieval, the slides were washed and incubated with blocking serum (0.1% bovine serum albumin in PBS plus 5% normal rabbit serum) for 1 hour followed by avidin-biotin blocking reagent. Incubation with a goat IgG polyclonal primary antibody against human RAGE (Catalog No. AF1145, R&D Systems, Minneapolis, MN) in a dilution of 1:400 or with blocking serum for secondary antibody control was performed overnight at 4°C. This was followed by incubation with a rabbit anti-goat secondary antibody (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and followed by incubation with ABC reagent (Vectastain ABC Kit, Vector Laboratories, CA). Finally, staining was completed utilizing DAB (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA). Staining reaction was stopped in distilled H_2O, and the slide was counter-stained with hematoxylin (Thermo Scientific, Waltham, MA).Evaluation of the stained slides was done by two blinded experienced investigators. In order to distinguish chromogen DAB from melanin pigment parallel sections stained lightly with H&E were used upon evaluation. Staining intensity was

analyzed based on pathological scoring as previously described.[25] In our study, the intensity 0 and 1 were combined and named "low intensity" and intensity 2 and 3 were combined and named "high intensity" (Supporting Information Figure S6). In case of discordant scoring results a consensus score was assigned. TMA spots with a lack of tumor tissue or crush artifacts were excluded from the analysis. Tissue samples were divided into sun-exposed vs. non sun-exposed based on the evaluation of severe collagen degeneration within the tissue punch or in the whole tissue specimen. Statistical significance was calculated by two-sided Fisher's exact test as it is recommended for sample sizes less than 1000.

Selection of polymorphisms and genotyping

The three single nucleotide polymorphisms (SNPs) -374T/A (rs1800624), -429T/C (rs1800625), and G82S (rs2070600) in the RAGE gene (AGER gene locus) were investigated because of either enhancing transcriptional activity (-374T/A and -429T/C) or showing enhanced ligand binding (G82S).[16,17] Human genomic DNA was obtained from the centrifuged blood cells of the EDTA blood samples from cohort 2 by using a kit from Qiagen (Valencia, CA, USA) according to manufacturer's instructions. Genotyping of the SNPs was done by using a PCR-based approach (RFLP) that had already been described and validated.[26] Briefly, for amplification of the region containing the two promoter SNPs (-374T/A and -429T/C), the following primers were used: forward primer 5'-GGGGCAGTTCTCTCCTCACT-3' and reverse primer 5'-GGTTCAGGCCAGACTGTTGT-3'. For amplification of the region in exon 3 containing the G82S SNP, 5'-GTAAGCGGGGCTCCTGTTGCA-3' was used as forward primer, and 5'-GGCCAAGGCTGGGGTTGAAGG-3' as reverse primer. For enzyme digestion of the resulting PCR products, the enzymes MfeI (for -374T/A) and AluI (for -429T/C and G82S) were utilized.

Statistics

Cut-off values for marker proteins were calculated utilizing a previously described algorithm of maximally selected statistics that accounts for multiple testing and thereby prevents from a large alpha error.[27] Based on data from the manufacturer of the RAGE ELISA that indicated

higher sRAGE levels in serum as compared to plasma, separate cut-off values for serum and plasma sRAGE were calculated. Cut-off values for sRAGE used in this study were 1012.07 pg/mL (serum) and 316.6 pg/mL (plasma). For serum esRAGE, a cut-off value of 321 pg/mL was calculated.

Estimates of cumulative overall survival probabilities according to Kaplan-Meier were compared using two-sided log-rank test statistics. Multivariate Cox proportional hazard analyses were used to evaluate the independent effects of sRAGE and the polymorphisms on OS. The models were stratified by stage to account for stage-dependent differences, and adjustment was made for age and sex of the patients. Results of the Cox models were described by means of hazard ratios (HRs) together with 95% confidence intervals (CIs), and *P* values were calculated based on the Wald test. Associations between serum markers were calculated by two-sided *Spearman's* rank correlation coefficient method. Receiver operating curve analyses were calculated using the R package 'pROC'.[28] Relative sRAGE concentrations were compared using two-sided Student's *t*-test.

Throughout the analyses, *P* values less than 0.05 were considered statistically significant. All analyses were carried out using R version 3.0.1 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

RAGE protein is abundantly expressed in malignant melanoma

Melanoma tissue-microarray analysis representing 35 primary melanomas and 41 associated benign nevi by immunohistochemistry using a specific antibody against RAGE revealed abundant expression of RAGE in melanoma and stromal cells but less expression in tissue of benign nevi ($p < 0.001$; Table 1 and Figure 1 A, B). Tissue samples from sun-exposed areas presenting signs of UV damage were associated with higher RAGE expression (Figure 1 D) in malignant melanoma ($p = 0.046$) but not in benign nevi ($p = 0.36$). Cutaneous UV damage is thought to play an important role in melanomagenesis.[31] For validation, immunohistochemical staining intensity of RAGE was analyzed based on a second independent tissue-microarray containing 257 primary melanomas and 22 benign nevi. Statistical analysis of the resulting pathological scoring again demonstrated a significant upregulation of RAGE protein expression in melanoma tissue as compared to benign nevi ($p = 0.005$; Table 1 and Figure 1 C).

Serum levels of soluble RAGE predict overall survival in stage III/IV melanoma patients

Previously, diminished levels of soluble forms of RAGE were identified in patients with chronic inflammatory diseases such as rheumatoid arthritis.[29] Moreover, sRAGE is thought to act as a decoy for its ligands and thereby to inhibit RAGE signaling. Based on these findings, we hypothesized that sRAGE levels diminish when the soluble receptor engages with one of its ligands e.g. with S100B which is abundantly expressed and released by melanoma cells. Therefore, serum of healthy donors was incubated with full-length bovine S100B protein with increasing concentrations and sRAGE levels were measured 24 hours after. Soluble RAGE levels diminished significantly in a S100B concentration-dependent manner (Supporting Information Figure S1). Therefore, we assumed that sRAGE might also be diminished in melanoma patients, especially in patients with elevated S100B levels, and thereby might bea potentially useful biomarker for disease progression. Subsequently, we measured soluble forms of RAGE (sRAGE and esRAGE) in the serum of an identification cohort (cohort 1) representing 229 stage III-IV melanoma patients (Table 2). As expected, by conducting a

small meta-analysis, we found that mean serum sRAGE was lower in the melanoma patients of cohort 1 compared to healthy individuals (Supporting Information Figure S2 A). Importantly, diminished serum sRAGE levels were identified as being significantly associated with impaired survival (p = 0.034; Figure 2 A). The endogenous splice variant esRAGE showed similar prognostic impact as sRAGE in cohort 1 (Figure 2 B). Moreover, sRAGE and esRAGE correlated significantly as well as ΔsRAGE (i.e. cleaved sRAGE only, representing the calculated difference between sRAGE and esRAGE) and esRAGE (Supporting Information Figure S3). Therefore, esRAGE measurement was not considered further in cohort 2. Receiver operating characteristic (ROC) analysis showed that the combination of S100B and sRAGE was able to predict OS better than S100B alone or S100B in combination with LDH (Figure 2 C). Moreover, multivariate Cox regression analysis including sRAGE and the established biomarkers S100B and LDH as well as stage and sex identified diminished sRAGE as an independent prognostic marker for impaired overall survival in two models with S100B and LDH, or LDH alone (Table 3). In the first model, LDH did not add independent prognostic information, which was probably due to its strong correlation with S100B (*Spearman's* rho = 0.46, p < 0.001, data not shown). Therefore, we performed Cox proportional hazards analysis without consideration of S100B (Table 2, model 2). In this second model, LDH and sRAGE correlated both independently with OS.

Diminished sRAGE is confirmed as a prognostic marker in melanoma patients

In an approach to validate the prognostic impact of sRAGE in the peripheral blood, we measured plasma levels of sRAGE in an independent second cohort (Table 2). Here, 173 stage I-IV melanoma patients were included for analysis. Median follow-up was 13.8 months. Again, mean plasma sRAGE was lower in these patients compared to healthy individuals (Supporting Information Figure S2 B). Diminished sRAGE levels were once more associated with impaired survival (p < 0.001). In cohort 2, patients with diminished plasma sRAGE levels had an unfavorable outcome with a 1-year survival rate of 46.9% whereas patients with normal plasma sRAGE levels did significantly better with a 1-year survival rate of 86.9% (p < 0.001) (Figure 3 A). Interestingly, plasma sRAGE levels stratified subgroups of stage IV

patients. M1c patients with diminished sRAGE levels showed drastically reduced overall survival (1-year survival rate 33.3%) compared to M1c patients with normal sRAGE levels (1-year survival rate 63.3%, log-rank p = 0.0052) (Figure 3 B). In contrast, the latter group of patients exhibited a comparable 1-year survival rate as M1a/b patients (63.3% vs. 59.2%, log-rank p = 0.94).

Genetic variants of RAGE may predict survival of melanoma patients

Genetic variants of RAGE have been associated with functional activity of the RAGE signaling pathway. In order to investigate for their association with survival of melanoma patients, we performed genotyping of three well-described functional SNPs in the RAGE gene (G82S, -374T/A, and -429T/C, Supporting Information Figure S4) using a PCR-based approach termed restriction fragment length polymorphism (RFLP) using genomic DNA obtained from peripheral blood cells of all EDTA blood samples from cohort 2. In our data set, no correlation between SNP status and sRAGE plasma levels was observed (G82S: p = 0.37, -374T/A: p = 0.87, -429T/C: p = 0.98) (Supporting Information Figure S5). Additionally, none single SNP was associated with OS (Supporting Information Table S1). However, multivariate Cox regression analysis revealed the presence of both promoter SNPs as an independent prognostic marker for impaired OS (p = 0.040, Supporting Information Table S1).

DISCUSSION

RAGE represents a central driver of tumorigenesis by sustaining an inflammatory tumor microenvironment.[6] Moreover, damage-associated molecular pattern molecules (DAMPs) and its receptors such as RAGE have gained further interest recently as the role of tumor-promoting inflammation has been identified as an attractive target of innovative anti-tumor therapies.[30,31] This study explores the importance of the DAMP receptor RAGE as a clinically useful prognostic biomarker of disease progression in melanoma patients.

Using two independent tissue-microarrays, our study revealed abundant expression of RAGE protein in malignant melanoma compared to benign nevi. RAGE expression has been detected in various types of cancer, e.g. colorectal, pancreatic and squamous cell carcinoma and its expression has been correlated with more aggressive types of cancer.[14,32-36] This supports previous findings of RAGE expression in various melanoma cell lines.[37,38] Moreover, genetic evidence in various mouse models has revealed RAGE as a central receptor that promotes inflammation-associated tumorigenesis.[6,39-41] In our study, RAGE protein expression was abundant in melanoma tissue compared to benign nevi underlining the importance of RAGE in melanomagenesis. Additionally, its high expression in melanomas with chronic UV damage sheds light on a possible role of RAGE signaling in the process of UV-associated melanoma development.[31]

In order to translate these findings to the clinical challenge of early detection of progressing melanoma patients, we evaluated the presence of circulating soluble forms of RAGE in serum/plasma of melanoma patients and its ability to predict overall survival. Using two independent patient cohorts, we demonstrated that diminished levels of a circulating soluble form of RAGE (sRAGE) in the peripheral blood of melanoma patients independently predict overall survival.

LDH and S100B are the most commonly used melanoma markers in clinical routine, and both have been shown to be reliable markers for survival of patients with distant metastasis (AJCC stage IV). Nevertheless, there is still a need for new biomarkers as the standard melanoma biomarker S100B fails to detect melanoma progression in up to 20% of patients.[5]

By including sRAGE, S100B, and LDH in a multivariate Cox proportional hazards model, we demonstrated that serum sRAGE independently provides additional prognostic information compared to the established biomarkers. Although sRAGE has not been studied in melanoma patients so far, diminished serum sRAGE levels have been observed in other forms of cancer (e.g. breast cancer[26], lung cancer[42], and pancreatic cancer[43]). Diminished levels of esRAGE were associated with a higher risk for developing pancreatic cancer within the first two years of follow-up.[44] In addition to these data, elevated levels of the RAGE ligands S100A8/A9 and HMGB1 have already been described in sera of cancer patients (e.g. colorectal, prostate, breast or oral squamous cell cancer).[45]

RAGE expression is ubiquitous and can be detected not only in melanoma cells but also in various immune cells and in endothelial cells.[7,8] sRAGE is released from these cells to the extracellular space and thought to consist of distinct variants of the receptor for advanced glycation end-products: predominantly (1) cleaved forms of membrane-bound full-length RAGE shed by metalloproteinases like ADAM10 and MMP9 - both abundant in the tumor microenvironment, (2) the endogenous splice variant esRAGE–representing approximately 20-25% of total sRAGE levels, and (3) other splice variant forms of RAGE.[8,9,1010,47] Moreover, previous studies were not able to detect full-length RAGE in the peripheral blood suggesting cellular damage or necrosis an unlikely way of release.[10] Otherwise, the quest for the cell-type specific source of sRAGE needs to be addressed by further studies. To evaluate whether the prognostic potential of total sRAGE is due to the cleaved form or the endogenous splice variant, we measured esRAGE in the serum samples of cohort 1. In our study, sRAGE and esRAGE levels seemed to be coordinately released to the peripheral blood as both markers correlated strongly. Additionally, a similar correlation is seen with esRAGE and cleaved sRAGE (= ΔsRAGE), i.e. sRAGE minus esRAGE (Supporting Information Figure S3). This supports recent studies demonstrating a strong similarity between total sRAGE and esRAGE levels.[11]

The correlation between diminished levels of sRAGE and impaired survival supports the hypothesis, that sRAGE serves as a decoy for RAGE activating ligands thereby blocking

cellular RAGE-RAGE ligand engagement and subsequently preventing cellular RAGE-mediated ~~membrane-bound RAGE and thereby inhibits RAGE~~ down-stream signaling.[7,15,46] sRAGE therefore could act as an intrinsic inhibitor of RAGE. Moreover, the S100B concentration-dependent decrease of sRAGE *in vitro* could at least partly explain diminished sRAGE levels in cancer patients and melanoma patients with impaired survival. It seems likely that RAGE-specific ELISA capture and detection antibodies are not able to bind RAGE molecules anymore when their binding sites are occupied by ligands. However, the S100B concentration at which sRAGE diminished *in vitro* was much higher than S100B concentrations one would expect in patients' sera. This is explained by the fact that RAGE ligands other than S100B are additionally upregulated in the serum of melanoma patients. Moreover, S100B concentrations could be much higher at the tumor site and in its microenvironment than in the peripheral blood. However, additional studies are required to clarify whether diminished sRAGE levels in melanoma patients with poor prognosis solely result from increasing levels of RAGE ligands such as S100B, S100A8/A9, or HMGB1, or whether they result from diminished release or enhanced down-regulation in melanoma or stromal cells to the benefit of enhanced expression of full-length RAGE. The latter hypothesis is supported by the notion that sRAGE levels add significant prognostic impact to the multivariate model independently of S100B, and that sRAGE serum levels did not correlate with S100B (*Spearman's* rho = -0.09, $p = 0.17$). However, the positive predictive value (PPV) of sRAGE alone was relatively low (Cohort 1: PPV = 0.380, Cohort 2: PPV = 0.474) possibly due to a relatively high number of high risk patients in cohort 1 (n = 100 for patients with low serum sRAGE compared to n = 129 for patients with high serum sRAGE) or a shorter follow-up period in cohort 2 leading to a biased PPV calculation. Despite these data, the combination of S100B and sRAGE was a better predictor of overall survival than S100B alone or S100B in combination with LDH (sRAGE and S100B: PPV = 0.826, S100B alone: PPV = 0.700, S100B and LDH: PPV = 0.762).

Upregulation of RAGE expression and release of soluble forms have been linked to functional genetic variants of RAGE[16,17] and naturally-occurring or acquired SNPs (single

nucleotide polymorphisms) of relevant genes have been implicated in melanoma pathogenesis.[48] Naturally-occurring, functional genetic variants within the AGER locus encoding for RAGE have been described recently. The SNPs -429T/C (rs1800625) and -374T/A (rs1800624) are situated in the AGER promoter region and have been shown to increase transcriptional activity of RAGE.[16-18] However, the functional role of these well-described SNPs within the genetic locus of RAGE has not been studied in melanoma patients so far. The variants of the two promoter SNPs (-374T/A, and -429T/C) showed a significant interaction and were associated with significantly impaired survival in a multivariate analysis, but not in univariate analysis (data not shown). Several reports have linked genetic variants of RAGE with an impaired prognosis of various cancer types e.g. gastric or lung cancer.[49,50] Moreover, the aforementioned findings highlight the importance of RAGE transcriptional activity in cancer and inflammation, since an association to these promoter SNPs is detected with higher disease activity of various inflammatory diseases such as rheumatoid arthritis or Crohn's disease.[19] Further evaluation of SNPs in the AGER locus using larger patient cohorts and extended genomic analyses will be required to clarify the role of genetic variants of RAGE in malignant melanoma.

Besides its role as prognostic marker in melanoma patients, RAGE signaling may serve as an attractive therapeutic target in melanoma. Possible strategies for inhibiting RAGE signaling using recombinant sRAGE, inhibitory antibodies against RAGE or its ligands, or small-molecule inhibitors have already demonstrated inhibitory effects on tumor growth in various mouse models.[34,37,46,51,52]

Despite its retrospective nature and restricted sample size, that limits subgroup analyzes, this study is the first to establish sRAGE as an independent prognostic blood-based marker for survival in melanoma patients with a high risk for progression. Together with the abundant expression of RAGE in malignant melanoma compared to benign nevi, this study supports the hypothesis that RAGE signaling might play an important role in melanoma. Further studies are required to validate these findings and to further investigate the implications of variants with its genetic locus on RAGE expression and prognosis of melanoma patients. In

conclusion, these data encourage the conduct of further experiments that focus on RAGE signaling as putative driving force of melanoma progression and on blocking strategies that may involve enhancement of sRAGE production as therapeutic option for melanoma therapy.

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REFERENCES

1. Villanueva J, Herlyn M. Melanoma and the tumor microenvironment. Current oncology reports 2008;10:439-46.

2. Eggermont AM, Spatz A, Robert C. Cutaneous melanoma. Lancet 2014;383:816-27.

3. Ugurel S, Utikal J, Becker JC. Tumor biomarkers in melanoma. Cancer control : journal of the Moffitt Cancer Center 2009;16:219-24.

4. Weide B, Elsasser M, Buttner P, Pflugfelder A, Leiter U, Eigentler TK, Bauer J, Witte M, Meier F, Garbe C. Serum markers lactate dehydrogenase and S100B predict independently disease outcome in melanoma patients with distant metastasis. British journal of cancer 2012;107:422-8.

5. Gebhardt C, Lichtenberger R, Utikal J. Biomarker Value and Pitfalls of Serum S100B in the Follow-Up of High-risk Melanoma Patients. JDDG. in press.

6. Gebhardt C, Riehl A, Durchdewald M, Nemeth J, Furstenberger G, Muller-Decker K, Enk A, Arnold B, Bierhaus A, Nawroth PP, Hess J, Angel P. RAGE signaling sustains inflammation and promotes tumor development. The Journal of experimental medicine 2008;205:275-85.

7. Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM, Nawroth PP. Understanding RAGE, the receptor for advanced glycation end products. Journal of molecular medicine (Berlin, Germany) 2005;83:876-86.

8. Kierdorf K, Fritz G. RAGE regulation and signaling in inflammation and beyond. Journal of leukocyte biology 2013;94:55-68.

9. Raucci A, Cugusi S, Antonelli A, Barabino SM, Monti L, Bierhaus A, Reiss K, Saftig P, Bianchi ME. A soluble form of the receptor for advanced glycation endproducts (RAGE) is produced by proteolytic cleavage of the membrane-bound form by the sheddase a disintegrin and metalloprotease 10 (ADAM10). FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2008;22:3716-27.

10. Hudson BI, Carter AM, Harja E, Kalea AZ, Arriero M, Yang H, Grant PJ, Schmidt AM. Identification, classification, and expression of RAGE gene splice variants. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 2008;22:1572-80.

11. Humpert PM, Djuric Z, Kopf S, Rudofsky G, Morcos M, Nawroth PP, Bierhaus A. Soluble RAGE but not endogenous secretory RAGE is associated with albuminuria in patients with type 2 diabetes. Cardiovascular diabetology 2007;6:9.

12. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. Journal of leukocyte biology 2007;81:1-5.

13. Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. Annual review of immunology 2010;28:367-88.

14. Gebhardt C, Nemeth J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. Biochemical pharmacology 2006;72:1622-31.

15. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, Neurath MF, Slattery T, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 1999;97:889-901.

16. Hudson BI, Stickland MH, Grant PJ, Futers TS. Characterization of allelic and nucleotide variation between the RAGE gene on chromosome 6 and a homologous pseudogene sequence to its 5' regulatory region on chromosome 3: implications for polymorphic studies in diabetes. Diabetes 2001;50:2646-51.

17. Hofmann MA, Drury S, Hudson BI, Gleason MR, Qu W, Lu Y, Lalla E, Chitnis S, Monteiro J, Stickland MH, Bucciarelli LG, Moser B, et al. RAGE and arthritis: the G82S polymorphism amplifies the inflammatory response. Genes and immunity 2002;3:123-35.

18. Pettersson-Fernholm K, Forsblom C, Hudson BI, Perola M, Grant PJ, Groop PH. The functional -374 T/A RAGE gene polymorphism is associated with proteinuria and cardiovascular disease in type 1 diabetic patients. Diabetes 2003;52:891-4.

19. Dabritz J, Friedrichs F, Weinhage T, Hampe J, Kucharzik T, Lugering A, Broeckel U, Schreiber S, Spieker T, Stoll M, Foell D. The functional -374T/A polymorphism of the receptor for advanced glycation end products may modulate Crohn's disease. American journal of physiology. Gastrointestinal and liver physiology 2011;300:G823-32.

20. Park SJ, Kleffmann T, Hessian PA. The G82S polymorphism promotes glycosylation of the receptor for advanced glycation end products (RAGE) at asparagine 81: comparison of wild-type rage with the G82S polymorphic variant. The Journal of biological chemistry 2011;286:21384-92.

21. Li K, Dai D, Zhao B, Yao L, Yao S, Wang B, Yang Z. Association between the RAGE G82S polymorphism and Alzheimer's disease. Journal of neural transmission (Vienna, Austria : 1996) 2010;117:97-104.

22. Kankova K, Vasku A, Hajek D, Zahejsky J, Vasku V. Association of G82S polymorphism in the RAGE gene with skin complications in type 2 diabetes. Diabetes care 1999;22:1745.

23. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, Buzaid AC, Cochran AJ, Coit DG, Ding S, Eggermont AM, Flaherty KT, et al. Final version of 2009 AJCC melanoma staging and classification. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2009;27:6199-206.

24. Bubendorf L, Nocito A, Moch H, Sauter G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol. 2001;195:72-9.

25. Ikenberg K, Fritzsche FR, Zuerrer-Haerdi U, Hofmann I, Hermanns T, Seifert H, Muntener M, Provenzano M, Sulser T, Behnke S, Gerhardt J, Mortezavi A, et al. Insulin-like growth factor II mRNA binding protein 3 (IMP3) is overexpressed in prostate cancer and correlates with higher Gleason scores. BMC cancer 2010;10:341.

26. Tesarova P, Kalousova M, Jachymova M, Mestek O, Petruzelka L, Zima T. Receptor for advanced glycation end products (RAGE)--soluble form (sRAGE) and gene polymorphisms in patients with breast cancer. Cancer investigation 2007;25:720-5.

27. Hothorn T, Lausen B. On the exact distribution of maximally selected rank statistics. Computational Statistics & Data Analysis 2003;43:121-37.

28. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Muller M. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC bioinformatics 2011;12:77.

29. Pullerits R, Bokarewa M, Dahlberg L, Tarkowski A. Decreased levels of soluble receptor for advanced glycation end products in patients with rheumatoid arthritis indicating deficient inflammatory control. Arthritis research & therapy 2005;7:R817-24.

30. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science (New York, N.Y.) 2013;339:286-91.

31. Bald T, Quast T, Landsberg J, Rogava M, Glodde N, Lopez-Ramos D, Kohlmeyer J, Riesenberg S, van den Boorn-Konijnenberg D, Homig-Holzel C, Reuten R, Schadow B, et al. Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in melanoma. Nature 2014;507:109-13.

32. Kuniyasu H, Oue N, Wakikawa A, Shigeishi H, Matsutani N, Kuraoka K, Ito R, Yokozaki H, Yasui W. Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer. The Journal of pathology 2002;196:163-70.

33. Hsieh HL, Schafer BW, Sasaki N, Heizmann CW. Expression analysis of S100 proteins and RAGE in human tumors using tissue microarrays. Biochemical and biophysical research communications 2003;307:375-81.

34. Arumugam T, Simeone DM, Schmidt AM, Logsdon CD. S100P stimulates cell proliferation and survival via receptor for activated glycation end products (RAGE). The Journal of biological chemistry 2004;279:5059-65.

35. Bhawal UK, Ozaki Y, Nishimura M, Sugiyama M, Sasahira T, Nomura Y, Sato F, Fujimoto K, Sasaki N, Ikeda MA, Tsuji K, Kuniyasu H, et al. Association of expression of receptor for advanced glycation end products and invasive activity of oral squamous cell carcinoma. Oncology 2005;69:246-55.

36. Tateno T, Ueno S, Hiwatashi K, Matsumoto M, Okumura H, Setoyama T, Uchikado Y, Sakoda M, Kubo F, Ishigami S, Shinchi H, Natsugoe S. Expression of receptor for advanced glycation end products (RAGE) is related to prognosis in patients with esophageal squamous cell carcinoma. Annals of surgical oncology 2009;16:440-6.

37. Abe R, Shimizu T, Sugawara H, Watanabe H, Nakamura H, Choei H, Sasaki N, Yamagishi S, Takeuchi M, Shimizu H. Regulation of human melanoma growth and metastasis by AGE-AGE receptor interactions. The Journal of investigative dermatology 2004;122:461-7.

38. Leclerc E, Heizmann CW, Vetter SW. RAGE and S100 protein transcription levels are highly variable in human melanoma tumors and cells. General physiology and biophysics 2009;28 Spec No Focus:F65-75.

39. Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J, Nguyen M, Olsson A, Nawroth PP, Bierhaus A, Varki N, Kronenberg M, et al. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. Carcinogenesis 2008;29:2035-43.

40. DiNorcia J, Lee MK, Moroziewicz DN, Winner M, Suman P, Bao F, Remotti HE, Zou YS, Yan SF, Qiu W, Su GH, Schmidt AM, et al. RAGE gene deletion inhibits the development and progression of ductal neoplasia and prolongs survival in a murine model of pancreatic cancer. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract 2012;16:104-12; discussion 12.

41. Kang R, Loux T, Tang D, Schapiro NE, Vernon P, Livesey KM, Krasinskas A, Lotze MT, Zeh HJ, 3rd. The expression of the receptor for advanced glycation endproducts (RAGE) is permissive for early pancreatic neoplasia. Proceedings of the National Academy of Sciences of the United States of America 2012;109:7031-6.

42. Jing R, Cui M, Wang J, Wang H. Receptor for advanced glycation end products (RAGE) soluble form (sRAGE): a new biomarker for lung cancer. Neoplasma 2010;57:55-61.

43. Krechler T, Jachymova M, Mestek O, Zak A, Zima T, Kalousova M. Soluble receptor for advanced glycation end-products (sRAGE) and polymorphisms of RAGE and glyoxalase I genes in patients with pancreas cancer. Clinical biochemistry 2010;43:882-6.

44. Grote VA, Nieters A, Kaaks R, Tjonneland A, Roswall N, Overvad K, Nielsen MR, Clavel-Chapelon F, Boutron-Ruault MC, Racine A, Teucher B, Lukanova A, et al. The associations of advanced glycation end products and its soluble receptor with pancreatic cancer risk: a case-control study within the prospective EPIC Cohort. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2012;21:619-28.

45. Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ, Geczy CL. Functions of S100 proteins. Current molecular medicine 2013;13:24-57.

46. Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Tanji N, Lu Y, Lalla E, Fu C, Hofmann MA, Kislinger T, et al. Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases. Nature 2000;405:354-60.

47. Yamamoto Y, Doi T, Kato I, Shinohara H, Sakurai S, Yonekura H, Watanabe T, Myint KM, Harashima A, Takeuchi M, Takasawa S, Okamoto H, et al. Receptor for advanced glycation end products is a promising target of diabetic nephropathy. Annals of the New York Academy of Sciences 2005;1043:562-6.

48. Gast A, Scherer D, Chen B, Bloethner S, Melchert S, Sucker A, Hemminki K, Schadendorf D, Kumar R. Somatic alterations in the melanoma genome: a high-resolution array-based comparative genomic hybridization study. Genes, chromosomes & cancer 2010;49:733-45.

49. Gu H, Yang L, Sun Q, Zhou B, Tang N, Cong R, Zeng Y, Wang B. Gly82Ser polymorphism of the receptor for advanced glycation end products is associated with an increased risk of gastric cancer in a Chinese population. Clinical cancer research : an official journal of the American Association for Cancer Research 2008;14:3627-32.

50. Wang X, Cui E, Zeng H, Hua F, Wang B, Mao W, Feng X. RAGE Genetic Polymorphisms Are Associated with Risk, Chemotherapy Response and Prognosis in Patients with Advanced NSCLC. PloS one 2012;7:e43734.

51. Saha A, Lee YC, Zhang Z, Chandra G, Su SB, Mukherjee AB. Lack of an endogenous anti-inflammatory protein in mice enhances colonization of B16F10 melanoma cells in the lungs. The Journal of biological chemistry 2010;285:10822-31.

52. Bjork P, Kallberg E, Wellmar U, Riva M, Olsson A, He Z, Torngren M, Liberg D, Ivars F, Leanderson T. Common interactions between S100A4 and S100A9 defined by a novel chemical probe. PloS one 2013;8:e63012.

FIGURE LEGENDS

Figure 1. (A) Representative microphotographs of a two independent tissue-microarrays containing primary melanomas and benign nevi analyzed by immunohistochemistry using a specific antibody against RAGE. Scale bars 100µm (lower magnification), 50µm (higher magnification). (B) Staining intensity of RAGE in 35 primary melanomas and 41 associated benign nevi of TMA 1, and (C) in 257 primary melanomas and 22 benign nevi of TMA 2 based on pathological scoring. (D) Staining intensity of RAGE in sun-exposed vs. non sun-exposed melanomas of TMA 1. Severe collagen degeneration was interpreted as sign of sun exposure. Statistical significance between patient numbers with low or high staining intensity was calculated by two-sided Fisher's exact test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Figure 2. Kaplan-Meier estimates of overall survival of the 229 stage III/IV melanoma patients from cohort 1 according to serum concentration of (A) sRAGE and (B) esRAGE. Cut-off values were 1012.07 ng/L for sRAGE and 321 ng/L for esRAGE. Statistical differences were calculated by two-sided log-rank test. (C) The predictive ability of the combination of S100B and sRAGE compared with S100B and LDH, and single markers by receiver operating characteristic (ROC) curves. The resulting AUC (area under the curve) values show that the combination of S100B and sRAGE was the best predictor of overall survival.

Figure 3. (A) Kaplan-Meier estimates of overall survival of the 173 stage I-IV melanoma patients from cohort 2 according to plasma concentration of sRAGE with a cut-off value of 316.6 ng/L. *P* value was calculated by two-sided log-rank test. (B) Kaplan-Meier estimates of overall survival of the 64 stage IV melanoma patients from cohort 2 according to M stage and plasma concentration of sRAGE. *P* value was calculated by two-sided log-rank test considering all stage IV patients.

Soluble RAGE and RAGE polymorphisms in melanoma

Table 1. Detailed description of the two independent tissue-microarrays.

Table 2. Patient characteristics at the time point of blood withdrawal for serum analysis.

Table 3. Multivariate Cox regression models for overall survival in cohort 1.

Supporting Information Figure S1. (A) Measured and (B) mean relative concentrations of sRAGE in human serum of healthy donors (n = 4) after incubation with bovine S100B at different concentrations. The resulting relative mean concentration of sRAGE showed a significant S100B concentration-dependent drop compared to mean relative sRAGE concentration at a resulting S100B concentration of 12.5 mg/L. Data shown in (B) are mean ± standard error of the mean (SEM) and statistical differences were calculated by two-sided Student's t-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

Supporting Information Figure S2. Meta-analysis of mean (A) serum sRAGE and (B) plasma sRAGE levels in healthy controls from all published articles between 2005 and 2013 containing at least 20 healthy controls from Caucasian ancestry and graphical comparison with mean sRAGE levels of the melanoma patients from cohorts 1 and 2. Melanoma patients' mean sRAGE levels are depicted as yellow bars, mean sRAGE levels of healthy controls are depicted as blue bars. Error bars represent standard error of the mean (SEM).

References:

Emanuele et al.: Arch Neurol 62:1734-6, 2005

Gaens et al.: J Clin Endocrinol Metab 94:5174-80, 2009

Gopal et al.: Eur J Clin Invest 42:807-14, 2012

Kalousova et al. (2006): Am J Kidney Dis 47:406-11, 2006

Kalousova et al. (2007): Nephrol Dial Transplant 22:2020-6, 2007

Krechler et al.: Clin Biochem 43:882-6, 2010

McNair et al.: Mol Cell Biochem 341:135-8, 2010

Pullerits et al.: Arthritis Res Ther 7:R817-24, 2005

Steiner et al.: Biol Psychiatry 65:1107-10, 2009

Tesarova et al.: Cancer Invest 25:720-5, 2007

Supporting Information Figure S3. Correlations between serum levels of (A) sRAGE and esRAGE, and (B) ΔsRAGE and esRAGE, calculated by two-sided Spearman's rank correlation coefficient. ΔsRAGE was calculated as the difference between measured sRAGE concentration and measured esRAGE concentration. sRAGE and esRAGE concentrations were measured in the 229 serum samples of cohort 1.

Supporting Information Figure S4. Schematic of three well-described SNPs (single nucleotide polymorphisms) within the AGER locus on chromosome 6 encoding for RAGE.

Supporting Information Figure S5. Correlations between SNP status and sRAGE plasma levels, calculated by two-sided Spearman's rank correlation coefficient.

Supporting Information Figure S6. RAGE protein expression in benign nevi (A-D) and primary melanomas (E-G) analyzed by immunohistochemistry: (A) Absent (B,E) weak, (C,F) moderate and (D,G) strong expression. (A) and (B,E) were considered "low intensity" and (C,F) and (D,G) "high intensity" RAGE protein expression. There was no primary melanoma lacking RAGE expression completely.

Supporting Information Table S1. Multivariate analysis of overall survival for RAGE polymorphisms. The combination of the promoter SNPs (single nucleotide polymorphisms) -374T/A and -429T/C was independently associated with impaired survival.

Table 1 – Wagner et al.

		TMA 1		TMA 2	
TMA characteristics		**n**	**%**	**n**	**%**
TMA Spots	Total spots	154	100.0	305	100.0
	Valid spots	152	98.7	279	91.5
	Missing spots	2	1.3	26	8.5
Patients#	No. of patients	**26**	**100.0**	**279**	**100.0**
	Mean follow-up [months (range)]	37.8 (2-73)		84.2 (1-186)	
Clinicopathological characteristics		**n**	**%**	**n**	**%**
Age	Mean [years (range)]	64.0 (43-90)		55.9 (14-89)	
Sex	Male	16	61.5	147	52.7
	Female	10	38.5	132	47.3
Histology	**Melanoma**	**35**	**100.0**	**257**	**100.0**
	SSM	19	54.3	203	78.9
	NMM	9	25.7	17	6.6
	LMM	1	2.9	25	9.7
	ALM	2	5.7	9	3.5
	not classifiable	4	11.4	3	1.3
T classification*	Tis	1	2.9	0	0
	T1	10	28.6	165	64.2
	T2	13	37.1	50	19.5
	T3	9	25.7	25	9.7
	T4	2	5.7	17	6.6
Ulceration	No	26	72.2	239	93.0
	Yes	10	27.8	18	7.0
Histology	**Benign nevi**	**41**	**100.0**	**22**	**100.0**
	compound	18	43.9	10	45.5
	junctional	9	22.0	5	22.7
	lentiginous	7	17.1	3	13.6
	dermal	6	14.6	3	13.6
	Nevus bleu	1	2.4	1	4.6
	not classifiable	0	0	0	0

Abbreviations: SSM, superficial spreading melanoma; NMM, nodular melanoma; LMM, lentigo maligna melanoma; ALM, acro-lentiginous melanoma.
*Tumor classification was performed according to the 2009 AJCC melanoma staging system of the American Joint Committee on Cancer.

Table 2 – Wagner et al.

Characteristic	Cohort 1		Cohort 2	
	No.	%	No.	%
No. of patients	229	100.0	173	100.0
Sex				
Female	99	43.2	75	43.4
Male	130	56.8	98	56.6
Age at Blood Withdrawal (mean)	59.6		60.5	
Range	16-88		24-84	
Localization of primary				
Extremities	109	47.6	65	37.6
Trunk	91	39.7	90	52.0
Unknown	29	12.7	18	10.4
Histological type of primary				
Superficial spreading	83	36.2	36	20.8
Nodular	51	22.3	35	20.2
Lentigo maligna	4	1.7	8	4.6
Acrolentiginous	30	13.1	9	5.2
Other or NA	26	11.4	74	42.8
Occult	35	15.3	11	6.4
Tumor staging*				
Stage I			34	19.7
IA			12	6.9
IB			22	12.7
Stage II			28	16.2
IIA			18	10.4
IIB			8	4.6
IIC			2	1.2
Stage III	160	69.9	47	27.2
IIIA	21	9.2	12	6.9
IIIB	82	35.8	21	12.1
IIIC	57	24.9	14	8.1
Stage IV	69	30.1	64	37.0
M1a	1	0.4	9	5.2
M1b	12	5.2	4	2.3
M1c	56	24.5	51	29.5

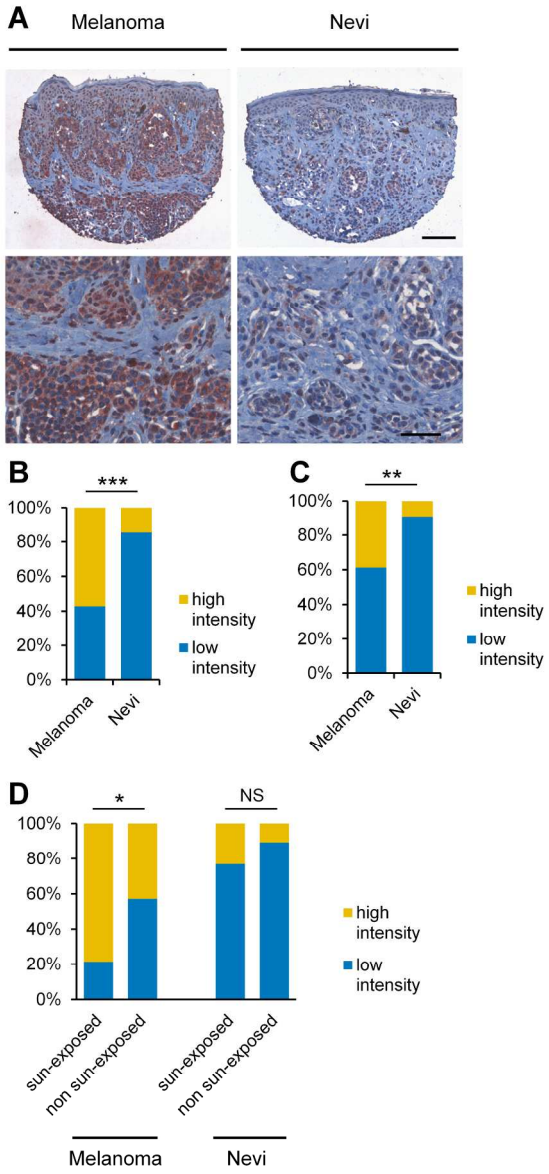
NOTE. Patient characteristics at the time point of blood withdrawal for serum analysis.
Abbreviations: NA, not applicable.
*Tumor staging was performed according to the 2009 AJCC melanoma staging system of the American Joint Committee on Cancer.

Table 3. Multivariate Cox regression models for overall survival in cohort 1

			Model 1		Model 2	
Prognostic factor	***n***	**% dead**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Stage of disease						
Stage III	160	15.0%	1		1	
Stage IV	69	66.7%	**6.1** (3.4-11.0)	**<0.001**	**9.3** (5.5-15.8)	**<0.001**
S100B						
normal	179	19.6%	1		Not considered	
elevated	50	70.0%	**3.4** (1.9-6.0)	**<0.001**		
LDH						
normal	197	26.4%	1		1	
elevated	32	56.3%	1.3 (0.7-2.4)	0.325	**2.1** (1.2-3.6)	**0.012**
sRAGE						
normal	129	24.8%	1		1	
diminished	100	38.0%	**1.9** (1.2-3.1)	**0.009**	**1.7** (1.0-2.7)	**0.035**

NOTE: Both models were adjusted for the confounding effects of sex. Significant results are shown in bold.
Abbreviations: CI, confidence interval; HR, hazard ratio.

Wagner et al.
Figure 1



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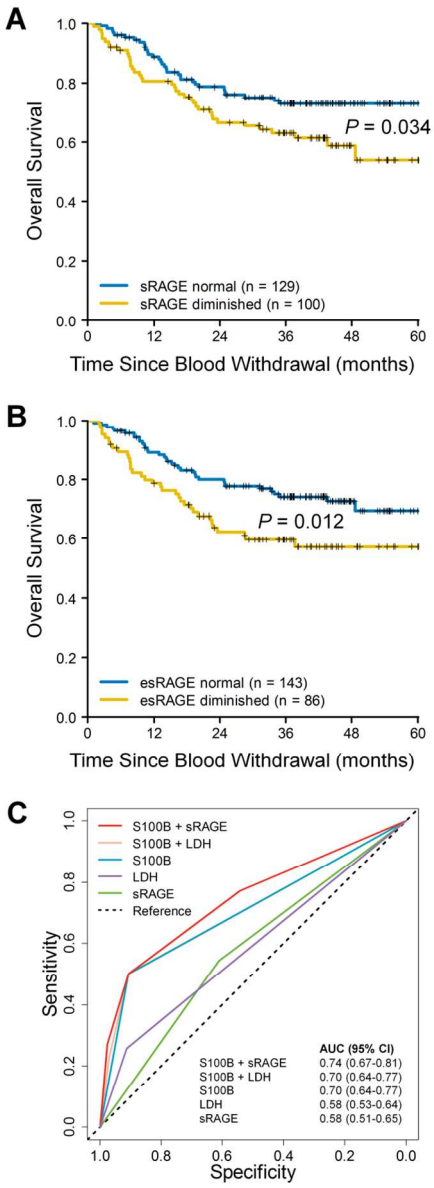

Wagner et al.
Figure 2

Figure 2. Kaplan-Meier estimates of overall survival of the 229 stage III/IV melanoma patients from cohort 1 according to serum concentration of (A) sRAGE and (B) esRAGE. Cut-off values were 1012.07 ng/L for sRAGE and 321 ng/L for esRAGE. Statistical differences were calculated by two-sided log-rank test. (C) The predictive ability of the combination of S100B and sRAGE compared with S100B and LDH, and single markers by receiver operating characteristic (ROC) curves. The resulting AUC (area under the curve) values show that the combination of S100B and sRAGE was the best predictor of overall survival.
114x152mm (300 x 300 DPI)

Wagner et al.
Figure 3

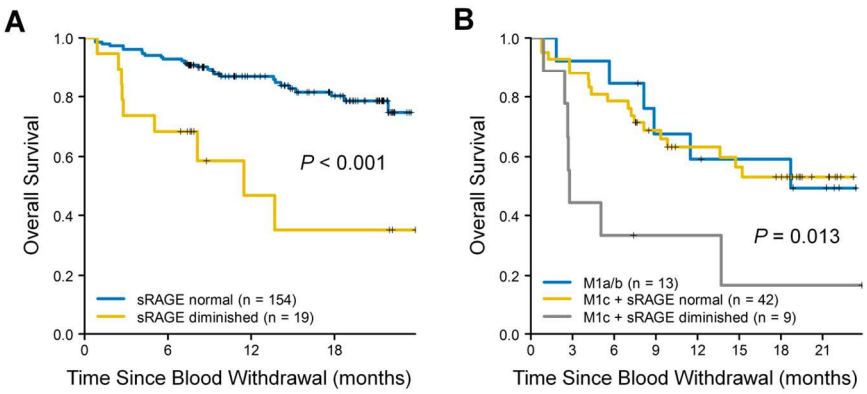

Figure 3. (A) Kaplan-Meier estimates of overall survival of the 173 stage I-IV melanoma patients from cohort 2 according to plasma concentration of sRAGE with a cut-off value of 316.6 ng/L. P value was calculated by two-sided log-rank test. (B) Kaplan-Meier estimates of overall survival of the 64 stage IV melanoma patients from cohort 2 according to M stage and plasma concentration of sRAGE. P value was calculated by two-sided log-rank test considering all stage IV patients.
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